Rinse and trickle: A protocol for TEM preparation and investigation of inorganic fibres from biological material

"Rinse and trickle": A protocol for TEM preparation and investigation of inorganic fibres from biological material

Ruggero Vigliaturo[1*], Silvana Capella[1,4], Caterina Rinaudo[2], Elena Belluso[1,3,5]

[11]Earth Sciences Department, University of Torino, via Valperga Caluso, 35-10125 Torino, Italy

[2]Science and Technological Innovation Department, University of Piemonte Orientale, via Teresa Michel, 11-15121 Alessandria, Italy

[3]CNR, Inst. of Geosciences and Earth Resources, via Valperga Caluso, 35-10125 Torino, Italy

[4]Centro Interdipartimentale per lo Studio degli Amianti e di altri Particolati Nocivi "G. Scansetti" - Università degli Studi di Torino, via Verdi, 8-10124 Torino, Italy

R. Vigliaturo: ruggero.vigliaturo@unito.it;

S. Capella: silvana.capella@unito.it; +39-011-6705135;

C. Rinaudo: caterina.rinaudo@unipmn.it; Tel. +39-0131-360256; Fax. +39-0131 360390

E. Belluso: elena.belluso@unito.it; Tel. +39-011-6705135; Fax. +39-011-6705128

Corresponding author

R. Vigliaturo: ruggero.vigliaturo@unito.it;

Present adress

School of Chemistry and Physics, University of KwaZulu-Natal, Durban, 4000, South Africa

Abstract

The purpose of this work is to define a sample preparation protocol that allows inorganic fibres and particulate matter extracted from different biological samples to be characterised morphologically, crystallographically and chemically by transmission electron microscopy-energy dispersive spectroscopy (TEM-EDS). The method does not damage or create artefacts through chemical attacks of the target material. A fairly rapid specimen preparation is applied with the aim of performing as few steps as possible to transfer the withdrawn inorganic matter onto the TEM grid. The biological sample is previously digested chemically by NaClO. The salt is then removed through a series of centrifugation and rinse cycles in deionised water, thus drastically reducing the digestive power of the NaClO and concentrating the fibres for TEM analysis. The concept of equivalent hydrodynamic diameter is introduced to calculate the settling velocity during the centrifugation cycles. This technique is applicable to lung tissues and can be extended to a wide range of organic materials. The procedure does not appear to cause morphological damage to the fibres or modify their chemistry or degree of crystallinity. The extrapolated data can be used in interdisciplinary studies to understand the pathological effects caused by inorganic materials.

Keywords: *inorganic fibres, organic material removal, TEM sample preparation protocol, centrifugation, settling velocity calculation, TEM-EDS investigation*

1. Introduction

There is a need for the detailed, reliable and unequivocal identification of inorganic fibres contained in biological samples, both to allow medical diagnostic evaluations and to enhance knowledge about the interactions between the biosphere and fibres. From an environmental point of view, it is fundamental to identify and prevent adverse health effects from asbestos, but it is also necessary to consider other natural and synthetic fibres with similar danger. First observations on asbestos fibres extracted from human lungs were performed by Ruska and Kuhn (Williams, 2009). From its origin, the observation of fibres revealed an interesting interaction between cells and the exogenous material, as evidenced by the observation of asbestos bodies by Kühn (1941). Currently, evidence of fibre transformation inside the lungs has been reported in a few papers dealing with transmission electron microscopy (TEM) or from in vitro studies (Germine, 2014), and earlier work often addressed the confirmation of the fibre type in environmental or work-related descriptions of asbestos, as requested by the World Health Organization criteria (WHO, 1986).

Given the incompletely understood mechanism of interaction among fibres and the organic environment, a detailed study of the possible chemical and morphological changes of fibres would be useful to unravel this knot. For this reason, it is necessary to use a preparation method that conserves the intrinsic characteristics, both chemical and morpho-dimensional, of the fibres without strongly perturbing the nature of the minerals.

Transmission electron microscopy (TEM) can provide direct measurement of particles and fibres extracted from cells and tissues after their reciprocal interaction. Many basic techniques for studying the state of the fibres after human body interactions have been presented by different authors, often generically describing certain fundamental steps and their own

consequences or just dealing in depth with a single aspect of the preparation (Webber et al. 2007). If we are really interested in the mineralogical evolution of the samples, precedence should not be given to particle or fibre loss but to absolutely not creating artefacts or damage that could confuse the interpretation of the mineralogical and biological phenomena.
The biological action of asbestos is not yet wholly clarified, and thus a unified sample preparation protocol that allows good analytical performance to be obtained in short time is fundamental.

This work aims to define a commensurate, reliable and unifying sample preparation protocol that allows the morphological, crystallographical and chemical characterisation of inorganic fibres and particulate matter extracted from different biological samples by TEM-energy dispersive spectroscopy (TEM-EDS). TEM is a powerful technique for the characterisation of individual particles and fibres that combines morphological, chemical and structural information (including the possibility of evaluating the increase and decrease of defects in the structure and the amorphisation or re-crystallization of the examined sample). This possibility allows a comparison between the characterization of the standard material in its natural condition and the changes in micro/nanofeatures of the fibres after close contact with the cellular and extracellular environment. The method used must avoid any damage to or artefacts in the investigated material caused by severe and incisive chemical attacks or mechanical stress. We present three variations of the same basic technique to better compare the advantages and disadvantages of slight variations in the different protocol steps.
A fairly rapid specimen preparation protocol is described with the aim of performing as few steps as possible to transfer the withdrawn inorganic matter onto the TEM grid. The biological sample is previously chemically digested by 13% NaClO in an oven at 60°C (Cook, 1979). After this, the salt must be removed from the suspension before it is dripped on

the Cu grid (the commonly used support) because it damages the carbon film coating and warps the mesh. The removal of the chemical digestive agent, carried out with a series of centrifugation and rinse cycles in deionized water, drastically reduces the solvent power of the NaClO. As well, the centrifugation concentrates the inorganic fibres and particulate matter, particularly in cases in which the original amounts are very low, and also prevents widespread loss due fibres clinging to the tube walls due to Van der Walls forces.

A theoretical approach for describing the settling velocity calculation of a material with a complex shape is also introduced using the concept of hydrodynamic equivalent volume to allow determination of the centrifugation time suitable for each expected fibre population.

2. Materials & methods

Two kinds of lung tissues and three different procedures were used to determine the best protocol for investigating inorganic fibres and particles extracted from biological material.

Lung tissues embedded in paraffin were obtained from autopsies. The tissues differed in terms of the fibre load: tissue with a low-concentration load (M_I) and tissue with a high-concentration load (C_I); the latter was used as a control. For testing of the different techniques, 4 samples of both specimen types were used. The procedures were always performed in duplicate to avoid the influence of the anomalous behaviour of a single particular sample (Table 1).

With particular attention to reducing the number of steps and the possibility of damaging the material, the first goal was to reduce the number of transfers of the fibre suspension across

several containers. In fact, from the first digestion of the chosen lung sample to the final withdrawal, the material never leaves the Falcon™ tube.

2.1 Sample set-up

Because lung tissue specimens were embedded in formalin, 0.1 g (weight as stored in the formalin) of tissue was recovered, gently dried with adsorbent paper and rinsed with deionized water.

2.2 Removal of the organic component

By always maintaining the parameters constant and varying only one parameter at a time, the amount of NaClO required for complete digestion that would result in a suspension as clear and transparent as possible and with no undigested material and mucilage was determined (Figure 1). In 10 mL of NaClO, 2 C_I samples and 2 M_I samples were digested in 4 trials. The same steps were performed in 20 mL of NaClO (Table 1). The process is detailed as follows.

a) The biological material was removed by chemical digestion directly in a Falcon™ tube by immersing 0.1 g of the tissue in 20 mL of 13% NaClO for about 1 day at 60°C (Belluso, 2006). If digestion was incomplete, 5 to 10 mL of NaClO was added, and the suspension was left to digest for another 6-12 hours at 60°C.

b) Deionized H_2O was added to bring the volume up to approximately 45 mL as the first dilution. This step is necessary both to reduce the digestive power of the NaClO and also to lower the viscosity of the suspension to allow the collection of as many fibres as possible along all surfaces of the tube walls.

In instances in which we found small remnants of a halo of mucilage (Figure 2), it was necessary to add 10 mL of digestive solution and continue the process for an additional 6-12 hours, which in most cases proved effective in eliminating all traces of organic material visible to the naked eye.

The digestion performed using 20 mL of NaClO with the subsequent addition of 10 mL after 24 hours was used as the basic step for all of the following procedures, thereby making it a constant within the experiment.

2.3 Recovery of inorganic fibres

c) The first step in recovering the inorganic fibres was centrifugation in accordance with the settling time calculated for the inorganic material under test, taking into account that it is advantageous to collect as much material as possible, by calculating the settling velocity of the smallest particles that are thought to be found.

d) After centrifugation, as much liquid as possible was removed with a pipette without disrupting the material at the bottom of the tube because this could remove material useful to the analysis.

e) The cycles of centrifugation and rinsing were repeated until the digestive power of the NaClO was eliminated. We have empirically estimated that the concentration of the digestive solution must be reduced by a factor at least of 200 to avoid damage to the Cu grid.

2.4 Transfer to the TEM support

f) Following these steps, a drop of the suspension was withdrawn from the bottom of the Falcon^{TM} tube and transferred onto the TEM grid, paying attention not to contact the pipette tip, the droplet and the grid simultaneously, to avoid removal instead of deposition of the material.

g) If few fibres were visible on the TEM grid through preliminary visual light microscopy observation, an additional droplet was added on the grid.

2.5 Investigation by TEM

The TEM investigation was performed on each mesh of the grid at the magnification useful for examining the morphological details of the fibres, usually between 20000 M and 60000 M.

For each revealed fibre, the following procedure was performed step by step: measure the fibre dimensions (length, diameter and ratio), control the crystalline state and, if crystalline, record at least one selected area diffraction pattern (SAED) along a main crystallographic direction, collect the energy dispersive spectrum and calculate the chemical composition with software. If the fibre contains oxygen, it is convenient to express the composition as oxide weight percent to compare it with data printed in the international literature (e.g. Wilson, 2013).

2.6 Identification of inorganic fibres by TEM-EDS

The fibres detected were classified on the strength of their dimensions as breathable and were operationally classified or not, according to the many current regulatory definitions (e.g. Centeno 2005; WHO 1986; Stanton, 1981).

Two main structural data are obtained from each recorded SAED: the distance between reticular planes, named d_{hkl} (in Å), and their reciprocal orientation. The procedure for measuring and elaborating data to obtain the d_{hkl} is explained in many books (e.g. Fultz and Howe, 2007).

The set of obtained data, both structural and compositional, can be used when searching for crystalline phase identification in a database (e.g. the PDF released from the International Centre for Diffraction Data, JCPDS, 1993) to identify the present analysed phases. For fibres having specific dimensions (i.e. length > 5 µm, width < 3 µm, length to width ratio < 3), a particular operational classification can be used (WHO, 1986).

.

3. Results and discussion

After defining the best NaClO quantity and the basic procedural steps, we tested three similar approaches to sample preparation (Figure 3), which we termed 'Rinse and Trickle' (R&T), 'Sparkle Rinse and Trickle' (SR&T) and 'Hydrogen Rinse and Trickle' (HR&T). All approaches were tested on both M_I and C_I samples.

The first approach, R&T, the simplest and speediest of the three, is detailed as in steps **a** to **g** above. The second approach, SR&T, involved the addition of two sonication steps, and the third approach, HR&T, involved the addition of hydrogen peroxide.

To test the three methods, we first used the sample with a high load of fibres (C_I), and then we tested the methods on the sample with the low load of fibres (M_I). This choice was made because the primary aim was not to evaluate the amounts of fibre for a statistical study but to

collect all of the material removed from the biological matter under the best possible conditions and without the preparation method causing any damage. The effective outcomes of the different approaches used were verified by TEM-EDS investigations combining imaging, diffraction and chemical analysis.

3.1 R&T approach

With this approach, different fibres were identified to be still trapped in organic material or to remain in different areas of the grid. This unsatisfactory result was verified for both the M_I and C_I samples.

3.2 SR&T approach

In the SR&T technique, two steps of ultrasonication were added, one during digestion and the other at the completion of digestion (step **a** above). The first ultrasonication comes after 6-8 hours of digestion, and the second is done just before the beginning of the centrifugation and rinse procedure (step **c** above). The outcome of this approach was verified for both samples M_I and C_I to show a low presence of organic material and mainly the total absence of organic clusters.

3.3 HR&T approach

The HR&T approach includes the addition of three drops hydrogen peroxide to the suspension of NaClO at 6-8 hours after the beginning of the digestion. The addition of H_2O_2, even though gently, inside the oven or during centrifugation, may provoke a reaction that leads to the loss of material and agitation of the suspension. This third technique could not be verified for any samples by TEM because of the almost total loss of material and the particular aggressiveness of the H_2O_2 against the layer of carbon on the TEM grid.

The digestions performed in 20 mL were more efficient than those in 10 mL. Moreover, the addition of NaClO after 24 hours improved the clarity of the suspension and reduced the presence of mucus and solid residues of visible size, in spite of the relatively short exposure time.

Considering the three approaches used to perform the sample preparation, the HR&T approach revealed that the reaction between the two liquids can be intense, especially if the second one is added quickly. If the suspension starts to foam and rise up, the possibility of material loss increases exponentially. The heterogenicity of the fibre population can also lead to the differential loss of material, limiting the characterization to the material less sensitive to physical events occurring inside the tube and which tends to remain undisturbed at the bottom of the tube. The TEM grid was most damaged by the drop placed during the HR&T approach. Many scraps of material were visible to the naked eye, and the amount of material for analysis was either absent or negligible and was found on the edges of the largely damaged grid.

The sample was well preserved and easy to pick up with the R&T procedure, but there was a strong presence of organic material of differing amounts covering the particles or fibres to be analysed. Owing to this coverage, many parts often appeared as a dark shapeless compound, diffraction investigations were hardly ever achievable and the chemical analyses were not attributable to specific material. In the M_I_d sample prepared by the R&T technique, we found long bundles of chrysotile trapped in massive pieces of organic material (Figure 4). There were even bundles less ‘polluted’ by organic material, but no bundle or single fibre observed in any of the C_I_d and M_I_d samples was totally free of organic matter (Figure 5). In the M_I_d sample, a single tremolite fibre (Figure 6) was found in to be in contact with a few

organic residues, whereas the chrysotile fibres were always wrapped in organic clusters. In the C_I_d sample, both the chrysotile fibres and amphibole fibres were not aggregated in bundles but were bonded singly with organic material.

From our analysis of the organic material containing fibres, it was evident that high concentrations of iron and silicon that were not recorded in the analysis of individual fibres or when analysing isolated organic material. It is therefore expected that in many of the fibres, residual ferruginous particles or other materials containing iron can probably also be found within these organic clusters.

The cleanliness of the fibres as evidenced by the lack of organic material was visibly improved with the SR&T approach. In fact, hardly any organic residues were observed or found to be in contact with the fibres (Figure 7), and none of the fibres or bundles were incorporated into organic clusters, thus allowing a better analytical performance. The cleanliness of the amphibole fibres is clearly visible in the analysed C_I_d sample shown in Figure 8. Further, in both the C_I_d and M_I_d samples, we regularly noticed the presence not only of magnesium but also of silicon in the area surrounding the fibres, as determined with chemical analysis by EDS. The detection of these elements was independent of the presence of organic material visible nearby the fibres.

The chrysotile fibres did not contain, of course, either sodium or chlorine, and their absence became a control element for the determination of a ‘good rinse’ of the fibres. Actually, no chemical analysis done in clean areas of chrysotile fibres free from organic material indicated the presence of chlorine or sodium.

4. Conclusions

A simple two-step basis for the preparation of fibre samples allows extreme and appreciable flexibility of application, in addition to the possibility of modifying and adapting the different steps in accordance with the diversity of possible biological samples. This technique permits the investigation of the possible transformations of exogenous inorganic materials that can occur during bio-interactions. The extrapolated data can be used within an interdisciplinary study (by mineralogists, pathologists, biologists or chemists) to focus on the opportunity to further understand the pathological effects caused by inorganic materials that penetrate into the human body.

With the illustrated protocol, we have

- avoided too many transfers of the starting material;
- reduced possible damage due to high temperature, ashing or strong chemical digestion;
- used a simple and flexible technique; and
- provided the mathematical basis for calculating the sedimentation rate of the fibrous material.

This protocol allows the morphological, chemical and crystallographic characterization of inorganic material after exposition and interaction with biologically active tissues and cells. It is the first step in accessing a field of study that allows the determination of the possible transformations that can occur during the interaction between organic and inorganic materials.

5. Appendix

Mathematical modelling for settling velocity

With the aim of calculating a value for the fibre settling velocity, a theoretical approach is necessary to introduce the concept of hydrodynamic equivalent volume; it will thus be possible to treat the fibres as spheres with the same density. This parameter, noted as D_{he}, refers to the diameter of a sphere with hydrodynamic behaviour identical to the fibre of interest. To determine an explicit formula for this quantity, we need to define the concepts of equivalent volume diameter, d_{ev}, and the dynamic shape factor, χ.

The first term, d_{ev}, represents the diameter of a sphere with volume identical to the fibrous particle considered (Kasper, 1982) for the settling velocity calculation. In the case of a fibre with a simplified cylindrical shape, d_{ev} may be derived from the following formula:

$$d_{ev} = d_f \sqrt[3]{\frac{3}{2}\beta}$$

where d_f is the diameter of the fibre, $\beta = \frac{L}{d_f}$, and L is the length of the fibre. In accordance with local shear stress and external force fields, a different orientation of the elongated particles occurs, with the theory of an ellipsoid in creeping motion applied to rod-like particles providing valuable insight into the effect of shape and orientation on the drag of non-spherical bodies. The factor χ may be regarded as a dimensionless constant that brings the drag force experienced by an irregularly shaped particle in relation to the equivalent volume diameter. For a cylindrical fibre that settles in a tube:

$$\frac{1}{\chi} = \frac{1}{3\chi_{\parallel}} + \frac{2}{3\chi_{\perp}}$$

If the fibre is oriented parallel to the settling direction, the dynamic shape factor is $\chi_{\parallel}$, whereas with the fibre oriented perpendicularly to the settling direction, $\chi_{\vdash}$ represents the dynamic shape factor:

$$\chi_{\parallel} = \frac{\frac{4}{3}(\beta^2-1)\beta^{-\frac{1}{3}}}{\{\frac{(2\beta^2-1)}{\sqrt{\beta^2-1}}\ln[\beta+\sqrt{\beta^2-1}]-\beta\}}$$

$$\chi_{\vdash} = \frac{\frac{8}{3}(\beta^2-1)\beta^{-\frac{1}{3}}}{\{\frac{(2\beta^2-3)}{\sqrt{\beta^2-1}}\ln[\beta+\sqrt{\beta^2-1}]+\beta\}}$$

Following the definition of all necessary parameters, it is possible to formulate D_{he} as follows:

$$D_{he} = \frac{d_{ev}}{\sqrt{\chi}}$$

To calculate the settling velocity of our spheres, we have to apply Stokes Law with a few corrections because of the acceleration due to the forces applied by the centrifuge. First, we define the speed of rotation in $\frac{radians}{s}$ as:

$$\omega = \frac{2\pi RPM}{60}$$

Then, we can define the centrifugal field named G:

$$G = \omega^2 r$$

where r is the distance of the considered equivalent sphere from the axis of rotation. After that, we can formulate the settling velocity as:

$$v_s = \frac{(\rho_f - \rho) D_{he}^2 \, G}{18\mu}$$

where ρ_f is fibre density, ρ is the density of the medium and μ is the viscosity of the fluid.

6. Acknowledgments

This research was conducted with the aid of a grant from the Italian National PROGETTO DI RILEVANTE INTERESSE NAZIONALE (PRIN) 2010–2011, prot. 2010MKHT9B 004, Research Unit 4, “Interazione fra minerali e biosfera: conseguenze per l’ambiente e la salute umana”.

7. References

Belluso E., Bellis D., Fornero E., Capella S., Ferraris G., Coverlizza S. (2006). Assessment of inorganic fibre burden in biological samples by SEM-EDS. Microchim. Acta, 155, 95-100.

Centeno J. A., Mullick F. G., Ishak K. G., Franks T. J., Burke A. P. (2005). Environmental Pathology. In Selinus O., Alloway B., Centeno J. A., Finkelman R. B., Fuge R., Lindh U., Smedley P. (Eds), Essentials of Medical Geology. Burlington, USA. pp. 563-594.

Cook P. M. (1979). Preparation of extra-pulmonary tissues and body fluids for quantitative transmission electron microscope analysis of asbestos and other mineral particle concentrations. Ann. N. Y. Acad. Sci., 330, 717-724.

Fultz B., Howe J. M. Transmission electron microscopy and diffractometry of materials, third edition. (Springer-Verlag, Heidelberg 2007).

Germine M., Puffer J. H. (2014). Analytical transmission electron microscopy of amphibole fibers from the lungs of Quebec miners. Arch. Environ. Occup. Health, 11, 0.

Kasper G. (1982). Dynamics and measurement of smokes. I Size characterization of non-spherical particles. Aerosol Sci. Technol., 1, 187-199.

JCPDS (1993). Mineral Powder Diffraction File Databook: Joint Committee on Powder Diffraction Standards. Swarthmore, PA, pp. 781.

Kühn J. (1941). Übermikroskopische Untersuchungen an Asbeststaub und Asbestlungen. Archiv für Gewerbepathologie und Gewerbehygiene, 10, 473-485.

Stanton M. F., Layard M., Tegeris A., Miller E., May M., Morgan E., Smith A. (1981). Relation of particle dimension to carcinogenicity in amphibole asbestos and other fibrous minerals. J. Natl. Cancer I. 67, 965-975.

WHO - Environmental Health Criteria 53. (1986). Asbestos and other natural mineral fibres. EHC 53, 1986. http://www.inchem.org/documents/ehc/ehc/ehc53.htm

Webber S. J., Czuhanich A. G., Carhart L. J. (2007). Performance of membrane filters used for TEM analysis of asbestos. J. Occup. Environ. Hyg., 4, 780-789.

Williams D. B., Carter C. B. (2009). Transmission Electron Microscopy: A Textbook for Materials Science. Springer, USA.